Potent in vitro Interference of Fleroxacin in DNA-binding, Unwinding and ATPase Activities of Bloom Helicase∗

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Abstract

Objective To study the effect of fleroxacin (FLRX) on biological properties of Bloom (BLM) helicase catalytic core (BLM642-1290 helicase) in vitro and the molecular mechanism of interaction between the two molecules.

Methods DNA-binding and unwinding activities of BLM642-1290 helicase were assayed by fluorescence polarization and gel retardation assay under conditions that the helicase was subjected to different concentrations of FLRX. Effect of FLRX on helicase ATPase activity was analyzed by phosphorus-free assay based on a colorimetric estimation of ATP hydrolysis-produced inorganic phosphate. Molecular mechanism of interaction between the two molecules was assayed by ultraviolet and fluorescence spectra.

Results The DNA unwinding and ATPase activities of BLM642-1290 helicase were inhibited whereas the DNA-binding activity was promoted in vitro. A BLM-FLRX complex was formed through one binding site, electrostatic and hydrophobic interaction force. Moreover, the intrinsic fluorescence of the helicase was quenched by FLRX as a result of non-radioactive energy transfer. The biological activity of helicase was affected by FLRX, which may be through an allostery mechanism and stabilization of enzyme conformation in low helicase activity state, disruption of the coupling of ATP hydrolysis to unwinding, and blocking helicase translocation on DNA strands.

Conclusion FLRX may affect the biological activities and conformation of BLM642-1290 helicase, and DNA helicase may be used as a promising drug target for some diseases.

Key words: Biological activity; BLM helicase; Fleroxacin; Interaction mechanism

INTRODUCTION

As an important member of the highly conserved DNA helicases found in organisms from bacteria and humans, the RecQ helicase family plays essential roles in genetic recombination, transcription, DNA replication, and DNA repair. Human RecQ helicases, such as Bloom (BLM) and Werner (WRN), play a key role in chromosomal maintenance, and

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their defects are associated with BLM syndrome (cancer predisposition) and WRN syndrome (premature aging predisposition)\textsuperscript{[3-4]}. BLM syndrome is a rare genetic disease caused by defects in BLM helicase of the RecQ family of helicases\textsuperscript{[5-6]}. The RecQ core of BLM helicase (amino acid residues 642-1290) displays the similar enzymatic properties of the full-size BLM helicase\textsuperscript{[7]}. As human RecQ helicases play a unique and important role in genomic stability, a number of strategies are explored to combat many types of cancer by modulating the functions of human RecQ helicases or their interaction factors\textsuperscript{[8-10]}. There is evidence that human RecQ helicases may serve as a suitable target for cancer therapeutics\textsuperscript{[8-9]}. Many small molecular drugs have proved that modulation of the expression level or function of the RecQ helicase is an efficient approach to cancer treatment\textsuperscript{[11]}. In addition, abnormal cancer cells are selectively regulated by inactivating the helicase-dependent DNA metabolic pathways including those for DNA recombination, replication, repair, and cell cycle checkpoint\textsuperscript{[12-13]}. Fleroxacin (FLRX, Figure 1) is an important antibiotic belonging to the class of drugs known as fluoroquinolones, and has been used in empirical treatment of a variety of infections, particularly infection of the genitourinary, gastrointestinal, and respiratory tracts\textsuperscript{[14]}. It was reported that fluoroquinolones can be used in treatment of cancers, such as bladder cancer\textsuperscript{[15]}. The functional mechanism of fluoroquinolones affects the activity of many related enzymes regulating DNA and energy metabolism in cells\textsuperscript{[16-18]}. It was demonstrated that FLRX affects the structure and function of topoisomerases II-IV, which are helpful for control of abnormal cells in diseases due to interference in DNA metabolism\textsuperscript{[19-20]}. The RecQ helicase is an efficient approach to cancer treatment\textsuperscript{[11]}. BLM syndrome (cancer predisposition) and WRN syndrome (premature aging predisposition)\textsuperscript{[3-4]}. BLM syndrome is a rare genetic disease caused by defects in BLM helicase of the RecQ family of helicases\textsuperscript{[5-6]}. The RecQ core of BLM helicase (amino acid residues 642-1290) displays the similar enzymatic properties of the full-size BLM helicase\textsuperscript{[7]}. As human RecQ helicases play a unique and important role in genomic stability, a number of strategies are explored to combat many types of cancer by modulating the functions of human RecQ helicases or their interaction factors\textsuperscript{[8-10]}. There is evidence that human RecQ helicases may serve as a suitable target for cancer therapeutics\textsuperscript{[8-9]}. Many small molecular drugs have proved that modulation of the expression level or function of the RecQ helicase is an efficient approach to cancer treatment\textsuperscript{[11]}. In addition, abnormal cancer cells are selectively regulated by inactivating the helicase-dependent DNA metabolic pathways including those for DNA recombination, replication, repair, and cell cycle checkpoint\textsuperscript{[12-13]}. Fleroxacin (FLRX, Figure 1) is an important antibiotic belonging to the class of drugs known as fluoroquinolones, and has been used in empirical treatment of a variety of infections, particularly infection of the genitourinary, gastrointestinal, and respiratory tracts\textsuperscript{[14]}. It was reported that fluoroquinolones can be used in treatment of cancers, such as bladder cancer\textsuperscript{[15]}. The functional mechanism of fluoroquinolones affects the activity of many related enzymes regulating DNA and energy metabolism in cells\textsuperscript{[16-18]}. It was demonstrated that FLRX affects the structure and function of topoisomerases II-IV, which are helpful for control of abnormal cells in diseases due to interference in DNA metabolism\textsuperscript{[19-20]}. 

**Figure 1. Chemical structure of FLRX.**

BLM helicase is associated with topoisomerase IIIα, telomerase-associated protein 1 (TEP1), heat shock protein 90 (HSP90), and topoisomerase IIα which performs specific tasks in cells\textsuperscript{[21-22]}. It was demonstrated that a positive super-coiled DNA appears when topoisomerase III is combined with human RecQ helicases, implying that the reverse gyrase activity of topoisomerase III (i.e. inducing positive coils) is affected\textsuperscript{[23]}, similar to that observed with Sgs1 and Top3 helicases in yeast\textsuperscript{[24]}. However, few studies are available on the interaction between FLRX and topoisomerases, and little information is available on the effect of FLRX on human DNA helicases. In the present study, the effect of FLRX on biological activity and conformation of BLM\textsuperscript{642-1290} helicase and its mechanism were evaluated, which may be beneficial for efficacy elucidation of DNA helicase as a drug target.

**MATERIALS AND METHODS**

**Reagents and Buffers**

All chemicals were of the reagent grade. All buffers were prepared using deionized water from a Milli-Q water purification system (Millipore Corp., USA) with a resistivity >18.2 MQ·cm. FLRX, ATP, and Hind III were purchased from Sigma (USA). ATPase activity assay kit was obtained from Innova Biosciences (England). Plasmid DNA purification kit was purchased from Beijing Dingguo Biological Technology Service Cooperation (China). The pH values were measured with an ORION pH meter and a 0079 microelectrode (ORION, USA), respectively, at 25 °C.

**BLM\textsuperscript{642-1290} Helicase**

The 6×his-tagged BLM\textsuperscript{642-1290} helicase was expressed in E. coli strain BL21 (DE3) via a pET15b expression plasmid as previously described\textsuperscript{[6]}. Briefly, the overexpressed helicase was purified at 4 °C by fast flow affinity chromatography on chelating Ni\textsuperscript{2+} with a sepharose column (GE Healthcare, USA), followed by FPLC size exclusion chromatography Superdex 200 (Amersham, Switzerland). Bromophenol blue-stained 10% SDS-PAGE analysis showed that the purity of BLM\textsuperscript{642-1290} helicase was over 95%.

**Oligonucleotides**

The sequences and lengths of DNA substrates are shown in Table 1\textsuperscript{[6,25-26]}. The PAGE-purified, fluorescein-unlabeled and labeled synthetic oligonucleotides were purchased from Shanghai Biological Engineering Technology Service Cooperation (China). Double-stranded DNA (dsDNA) was obtained from DNA hybrid buffer (20 mmol/L Tris-HCl, 100 mmol/L NaCl, and pH 7.4) by adding an
equal amount of 2 types of single-stranded DNA (ssDNA). The mixture was heated to 85 °C for 5 min, and annealed by slow cooling to room temperature. The duplexes were used as DNA substrates in fluorescence polarization assays.

The plasmid DNA (3 kilobase) was purified with a plasmid DNA purification kit. Linear PMD-18T dsDNA was produced by incubating plasmid DNA with Hind III in water at 37 °C for 3 h. The concentration of dsDNA was measured with an ultraviolet (UV) spectrophotometer (GE Healthcare, USA) equipped with a 1.0 cm cuvette.

**DNA-binding Activity Assay**

The DNA-binding activity was assayed with a Beacon 2000 fluorescence polarization instrument (Pan Vera Corp., USA)[25]. Two nmol/L fluorescein-labeled dsDNA (A1A2) or ssDNA (A2) was added into the reaction buffer (20 mmol/L Tris-HCl, 20 mmol/L NaCl, 3 mmol/L MgCl₂, 0.1 mmol/L dithiothreitol at pH 7.9) in a temperature-controlled cuvette at 25 °C. The anisotropy was measured immediately until it was stabilized. BLM²⁴²⁻¹²⁹⁰ helicase was subjected to FLRX stress at different concentrations for 2 min, and the miscible liquid was added into the cuvette (a total volume of 150 mL). The change in the sample was immediately assayed. The dissociation binding constants (apparent $K_d$ values) were calculated in the presence of FLRX following equation (1) and equation (2) as previously described[25].

$$\alpha D_T = N P_r \frac{\alpha}{\alpha + K_d} + K_d \tag{1}$$

$$\alpha = \frac{A_{\text{max}} - A}{A_{\text{max}} - A_{\text{min}}} \tag{2}$$

Where $D_T$ is the total molar concentration of DNA and $P_r$ is the helicase used, $A$ is the fluorescence anisotropy at a given concentration of helicase, $A_{\text{max}}$ is the anisotropy at saturation, and $A_{\text{min}}$ is the initial anisotropy.

In addition, the interaction between FLRX and BLM-DNA complex was assayed. Two nM fluorescein-labeled dsDNA or ssDNA was titrated with helicase until saturated at 25 °C and the complex was titrated with 100 μmol/L FLRX. The changes in anisotropy were recorded every 8 sec until stabilized.

**DNA Unwinding Activity Assay**

DNA unwinding activity was quantified as previously described[26]. Two nM fluorescein-labeled dsDNA was added into the reaction buffer in a temperature-controlled cuvette at 25 °C. The anisotropy of DNA substrates was recorded at an interval of 8 sec, until it was stabilized. The BLM²⁴²⁻¹²⁹⁰ helicase was subjected to various concentrations of FLRX for 2 min, followed by adding the miscible liquid into the cuvette. The change in anisotropy was immediately assayed until it was stabilized. One mM ATP was added into the cuvette (a total volume of 150 mL) and the changes in anisotropy were recorded every 8 sec until it was stabilized.

**ATPase Activity Assay**

The ATPase activity was assayed with an ATPase activity assay kit based on a colorimetric estimation of ATP hydrolysis-produced inorganic phosphate[27]. The dilute helicase was subjected to FLRX stress at different concentrations for 2 min, and the reactions were initiated by adding it into the ATPase activity assay buffer [0.5 mol/L Tris at pH 7.9, with 10 mmol/L ATP, 4 nmol/L ssDNA (B1), and 0.1 mol/L MgCl₂ at 25 °C]. The reacting mixture (200 μL) was removed at different time points (2.5, 5, 7.5, 10, 15, 20, 25, and 30 min), and rapidly mixed with 50 μL Gold mix to terminate the ATP hydrolysis. The samples were allowed to stand for 2 min, and 50 μL stabilizer was added into each sample. The OD₅₅₀ of the sample was measured by UV spectrophotometry after 30 min. The enzyme activity $A_{\text{activity}}$ (units-mL⁻¹-min⁻¹) was defined as one unit of enzyme required to catalyze the reaction of 1 μmol/L substrate per minute at 25 °C. The ATPase activity of

**Table 1. Oligonucleotide Sequences of DNA Substrates Used in this Study**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Length/mer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>45</td>
<td>5′-AATCCGTCGACAGATGGTTAGGTAGGTTAGTTTTTTTTT-3′</td>
</tr>
<tr>
<td>A2</td>
<td>21</td>
<td>3′-FAM-TTAGCCGCTCGTCTCAATCC-5′</td>
</tr>
<tr>
<td>B1</td>
<td>20</td>
<td>5′-TGGACCATCGTTTTTTTTTTT-3′</td>
</tr>
</tbody>
</table>

Note. *FAM* represents the fluorescent chemical group.
undiluted enzyme was assayed following equation (3) according to the kit instructions.

\[ A_{\text{activity}} = \frac{A \times C}{500 B} \]  

(3)

Where \( A \) is the concentration of Pi (\( \mu \)mol/L) measured from the standard curve, \( B \) is the assay time (minute), and \( C \) is the reciprocal of enzyme dilution factor.

Steady-state kinetic parameters (\( K_m \) and \( K_{cat} \) for ATP) were detected with 10 nmol/l helicase in the presence of 0, 40, and 100 nmol/mL of FLRX. The ATP concentration was 0.1-1 mmol/L. The \( K_m \) and \( K_{cat} \) values were derived from the Michaelis-Menten equation for the reactions.

**Agarose Gel Mobility Shift Assay**

The binding and unwinding activities of the helicase were confirmed by agarose gel mobility shift assay. BLM-DNA complexes were formed in 25 \( \mu \)L reaction buffer at room temperature. The 60 \( \mu \)mol/L Hind III-restricted 3-kilobase dsDNA was mixed with 10 \( \mu \)mol/L helicase at different concentrations of FLRX (0-600 \( \mu \)mol/L). Five \( \mu \)L loading buffer (40 nmol/L Tris acetate, pH 7.9, 50% glycerol, and 0.25% (w/v) bromophenol blue) was added into each sample following incubation for 30 min. The complexes were separated by electrophoresis through 0.8% agarose gel in TAE buffer (40 mmol/L Tris acetate, 1 mmol/L EDTA, pH 8.3) at 100 V for 1 h. The bands of complexes were visualized with Gold view staining in UV light. The unwinding activity of helicase was assayed by adding 10 nmol/L ATP and 10 \( \mu \)mol/L helicase into the mixture of 60 \( \mu \)mol/L dsDNA at different concentrations of FLRX (0-600 \( \mu \)mol/L).

**UV Absorption Spectra Assay**

The UV absorption spectra of BLM helicase were detected by UV spectrophotometry. The spectra of 2 \( \mu \)mol/L helicase were analyzed with enzyme-free Tris-HCl buffer (pH 7.9) as the control. Then, Different concentrations of FLRX (0, 1, 3, 4, 5, 6, 7, 9, 10, 12.5, 15, and 20 \( \mu \)mol/L) were added into the Tris-HCl buffer. The sample was incubated for 30 min before assaying. The spectra of FLRX, helicase, and mixture of FLRX and helicase were recorded, respectively.

**Fluorescence Spectra Assay**

The fluorescence spectra were measured with a Cary eclipse spectrofluorometer (Hewlett-Packard, USA) equipped with a xenon lamp source and 1.0 cm cells. The fluorescence spectra of helicase were detected in Tris-HCl buffer (pH 7.9) when the width of entrance and exit slit was 5 nm and the scanning speed was 750 nm/sec. The 10 \( \mu \)mol/L helicase was mixed with different concentrations of FLRX (0, 1, 2, 3, 5, 7, 9, 11, and 13 \( \mu \)mol/L) in the Tris-HCl buffer (a total accumulated volume of 400 \( \mu \)L, pH 7.9). The reactions were assayed at 4, 25, and 40 °C. The type of fluorescent quenching was determined following the equation (4) as previously described \[28\].

\[ F_0/F = 1 + K_{q0}[Q] = 1 + K_{q0}[Q] \]  

(4)

Where \( F_0 \) is the fluorescence intensity in the absence of quencher, \( F \) is the fluorescence intensity in the presence of quencher concentration, \( K_{q0} \) is the quenching rate constant of biomacromolecule, \( K_w \) is the quenching rate constant of dynamic quenching and \([Q]\) is fluorescence lifetime of biomacromolecule without quencher. \( \tau_0 \) is usually about 10⁻⁸ sec \[28-29\]. The maximal collisional quenching rate constant was 2.0×10¹⁰ L-mol⁻¹·s⁻¹ for all classes of the biomolecule \[30\].

The binding constant and the number of binding sites were detected following equation (5) as previously described \[29-30\].

\[ \lg ([F_0-F]/F) = lg K + n lg [Q] \]  

(5)

Where \( K \) is the binding constant and \( n \) is the number of binding sites.

The acting forces include hydrogen bonds, Vander Waals forces, electrostatic forces, and hydrophobic interaction forces between small molecule drugs and biomacromolecules. The reaction enthalpy change is regarded as a constant when the temperature is almost not changed. Thus, the types of acting forces can be determined following equations (6 and 7) as previously described \[17\].

\[ \ln K = - \frac{\Delta H}{RT} + \frac{\Delta S}{R} \]  

(6)

\[ \Delta G = \Delta H - T \Delta S \]  

(7)

Where \( K \) is the binding constant, \( T \) is the temperature, \( R \) is the gas molecule constant, \( \Delta H \) is the binding enthalpy change, \( \Delta S \) is the binding entropy change, \( \Delta G \) is the Gibbs’ free energy change.

The acting force was hydrophobic interaction force, hydrogen bonds and Vander Waals force, and electrostatic force, respectively, when \( \Delta H>0 \) and \( \Delta S>0 \), \( \Delta H<0 \) and \( \Delta S<0 \), and \( \Delta H=0 \) and \( \Delta S>0 \) \[30-31\].

According to the Förster non-radioactive energy transfer theory [equations (8)-(10)] \[29-30\], the energy-transfer relation is related not only to the distance between acceptor and donor (r), but also to...
the critical energy transfer distance (with a transfer efficiency of 50%, $R_0$).

$$E = R_0^6/(R_0^6 + r^6)$$

(8)

$$E = 1 - F/F_0$$

(9)

$$R_0^6 = 8.8 \times 10^{-25} K^2 N^{-1} \phi J$$

(10)

Where $K^2$ is the spatial-orientation factor of dipole, $N$ is the refractive index of medium, $\phi$ is the fluorescence quantum yield of donor, $E$ is the energy transfer effect, $F$ is the fluorescence intensity when $C_{(FLRX)/C_{(BLM)}}=1:1$. In this study, $K^2 = 2/3$, $N=1.336$, and $\phi = 0.118^{[31]}$. $J$ is the overlap integral of the fluorescence emission spectrum of donor and the absorption spectrum of acceptor when $C_{(FLRX)/C_{(BLM)}}=1:1$ [equation (11)].

$$J = \left[ \sum I_D(\lambda) \varepsilon_D(\lambda) \Delta \lambda \right] / \left[ \sum I_D(\lambda) \Delta \lambda \right]$$

(11)

Where $I_D(\lambda)$ is the fluorescence intensity of fluorescent donor at wavelength $\lambda$, and $\varepsilon_D(\lambda)$ is the molar absorbance of acceptor at wavelength $\lambda$.

**Statistical Analysis**

The data were analyzed by F-test and SPSS 13.0 (SPSS 13.0, 2005, SPSS Inc., Chicago, USA) according to the general linear model. $P<0.05$ was considered significant and $P<0.01$ was considered extremely significant. The data are expressed as mean±SD.

**RESULTS**

**Effects of FLRX on DNA-binding Activity of BLM642-1290 Helicase**

The effect of FLRX on DNA-binding activity of BLM642-1290 helicase was shown in Figure 2. In terms of anisotropy, when the FLRX reacted directly with helicase and the BLM-DNA complex reacted with either ssDNA (Figure 2A) or dsDNA as a substrate (Figure 2B), the anisotropy was enhanced with the increasing FLRX concentration ($P<0.01$). In case of direct reaction with helicase, the anisotropy of FLRX was higher ($P<0.05$) than that of the BLM-DNA complex with ssDNA and dsDNA as substrates. When the ssDNA and dsDNA were used as substrates, an extremely significant difference was found in the reaction as FLRX reacted with helicase ($P<0.01$). As a substrate, the anisotropy of ssDNA was higher than that of dsDNA, revealing that the effect of FLRX on DNA-binding activity is related to the substrate structure. These results (Figure 2C) showed that the anisotropy of ssDNA and dsDNA escalated with the increasing FLRX concentration ($P<0.01$), which was lower when the FLRX reacted with helicase and BLM-DNA complex, indicating that the FLRX promotes the DNA-binding activity of helicase in a concentration-dependent manner. The variation in $K_d$ values was analyzed at different concentrations of FLRX (Figure 3), which showed that the $K_d$ values...
decreased with the increasing FLRX concentration \((P<0.01)\), suggesting that the FLRX promotes the molecular affinity of helicase and DNA.

In order to further confirm these results, the effect of FLRX on DNA-binding activity of helicase was analyzed by agarose gel mobility shift assay (Figure 4), which demonstrated that the amount of BLM-DNA complex increased with the increasing FLRX concentration and the corresponding amount of unbound dsDNA decreased, both were consistent with the results of fluorescence polarization assay.

**Effect of FLRX on DNA Unwinding Activity of BLM\textsuperscript{642-1290} Helicase**

The effect of FLRX on DNA unwinding activity of BLM\textsuperscript{642-1290} helicase is shown in Figure 5. The anisotropy of unwinding remarkably decreased with the increasing FLRX concentration \((P<0.01)\), indicating that FLRX effectively inhibits the DNA unwinding activity in a concentration-dependent manner (Figure 5A). Fluorescence polarization and agarose gel mobility shift assay showed that FLRX could not completely inhibit the DNA unwinding activity. The half inactivated concentration \((C)\) of FLRX for DNA unwinding activity was 60.5±10.2 μmol/L (Table 2). One hundred and fifty seconds were necessary for helicase unwinding with dsDNA as a substrate at different FLRX concentrations (Figure 5B). However, the DNA unwinding extent was significantly different \((P<0.01)\) at different FLRX concentrations.

**Table 2. Effect of FLRX on ATPase Activity Constants of BLM\textsuperscript{642-1290} Helicase**

<table>
<thead>
<tr>
<th>BLM\textsuperscript{642-1290}</th>
<th>ATPase Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>(V_{\text{max}}) (μmol/L·min(^{-1}))</td>
</tr>
<tr>
<td>4 nmol/L FLRX</td>
<td>118.4±8.6**</td>
</tr>
<tr>
<td>10 nmol/L FLRX</td>
<td>68.5±4.9**</td>
</tr>
<tr>
<td>100 nmol/L FLRX</td>
<td>18.8±1.6**</td>
</tr>
</tbody>
</table>

**Note.** *P<0.05,* **P<0.01** vs control group. Data are expressed as mean±SD.
**Effect of FLRX on ATPase Activity of BLM**

FLRX significantly affected the ATPase activity of helicase ($P<0.05$) (Figure 6A). The $A_{activity}$ of helicase decreased with the increasing reaction time and the FLRX concentrations, suggesting that FLRX inhibits the $A_{activity}$ of helicase in a time- and dependent manner ($P<0.01$, Figure 6B). The $C_i$ value of FLRX for ATPase activity was $10.3\pm3.1$ μmol/L (Table 2). However, the ATPase activity was not inhibited by FLRX even at the concentration of 100 μmol/L. The $K_m$ was invariable, the $V_{max}$ decreased with the increasing FLRX concentrations, the $K_{cat}$ decreased with $V_{max}$ (Table 3), demonstrating that FLRX cannot inhibit the ATPase activity of helicase.

**Figure 6.** Time-dependent (A) and concentration-dependent (B) inhibitory effect of FLRX on ATPase activity of BLM helicase.

**Effect of FLRX on UV Absorption Spectra of BLM Helicase**

The effect of FLRX on UV absorption spectra of helicase is shown in Figure 7. The UV spectra of FLRX, the helicase, and their mixture showed different maximum absorption peaks, suggesting that FLRX may form a BLM-FLRX complex by reacting with helicase (Figure 7A). The UV absorption values of helicase increased with the increasing FLRX concentrations. However, the changes in absorption peaks were non-significant ($P<0.05$) at the tested concentrations of FLRX. The FLRX concentrations were lower than 7.0 μmol/L (Figure 7B), and the maximum absorption of the complex was found at 238 nm. However, the concentration was beyond the bounds of 7.0 μmol/L, and the maximum absorption varied from 238 to 280 nm (Figure 7C), demonstrating that FLRX can affect the conformation of helicase.

**Effect of FLRX on Fluorescence Spectra of BLM Helicase**

The effect of FLRX on fluorescence spectra of BLM helicase was shown in Figure 8. The excitation and emission wavelengths were 275 nm and 305 nm, respectively. The fluorescence intensity of helicase decreased with the increasing FLRX concentrations, indicating that FLRX can quench the intrinsic fluorescence of helicase. The $K_q$ values were $6.76\times10^{14}$, $5.67\times10^{14}$, and $4.72\times10^{14}$ L·mol$^{-1}$·s$^{-1}$ at 4 °C, 25 °C, and 40 °C, respectively, which were higher than the maximal collisional quenching constants for all classes of the biomolecule (Table 3 and Figure 9), indicating that fluorescent quenching is caused by dynamic collisions between helicase and FLRX rather than by
static quenching.

**Figure 8.** Effect of FLRX on fluorescence spectra of BLM

![Image of fluorescence intensity vs wavelength for BLM helicase with FLRX at different temperatures.](image)

**Figure 9.** Stern-Volmer curves for fluorescence quenching of BLM helicase by FLRX at different temperatures.

The $K$ values were $6.982 \times 10^6$, $4.842 \times 10^6$, and $3.597 \times 10^6$ L·mol$^{-1}$ at 4 °C, 25 °C, and 40 °C, respectively (Figure 10 and Table 4), indicating that there is a strong binding force between helicase and FLRX. The $n$ values were 0.9965, 0.9941, and 0.9973 respectively at 4 °C, 25 °C, and 40 °C, suggesting that the helicase and FLRX can form a BLM-FLRX complex at one binding site.

**Table 3.** Stern-Volmer Equations and Quenching Constants for Fluorescence Quenching of Helicase by FLRX at Different Temperatures

<table>
<thead>
<tr>
<th>Quenching agent</th>
<th>T/°C</th>
<th>$F_0/F$-[Q] equation</th>
<th>$K$ ($\times 10^4$ L·mol$^{-1}$·s$^{-1}$)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLRX</td>
<td>4</td>
<td>$y=6.76 \times 10^3 x+1.0096$</td>
<td>6.76</td>
<td>0.9954</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>$y=5.67 \times 10^3 x+0.9899$</td>
<td>5.67</td>
<td>0.9970</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>$y=4.72 \times 10^3 x+0.9930$</td>
<td>4.72</td>
<td>0.9987</td>
</tr>
</tbody>
</table>

**Figure 10.** Plot of log ($F_0/F$) vs log [Q] at different temperatures.

The $\Delta H$, $\Delta G$, and $\Delta S$ of the reaction between helicase and FLRX are shown in Figure 11 and Table 5. The $\Delta G$ was smaller than zero at 4 °C, 25 °C, and 40 °C, indicating that the reaction between helicase and FLRX occurs spontaneously. The contribution of $\Delta S$ to $\Delta G$ was greater than that of $\Delta H$ to $\Delta G$, demonstrating that the interaction acting forces are electrostatic force and hydrophobic interaction force.

The overlap between the FLRX absorption spectrum of and the helicase fluorescence emission spectrum was shown in Figure 12. The overlap integral ($J$) could be assessed by integration with the Metlab program. $J = 2.15 \times 10^{-14}$ cm$^3$·L·mol$^{-1}$, at 25 °C, $E$ was 0.254, at 25 °C, $R_0$ and $r$ were 4.09 nm and 4.89 nm respectively. The $E$, $R_0$, and $r$ at 4 °C and 40 °C are listed in Table 4. It was reported that non-radioactive energy transfer occurs between the donor and receptor when the distance between two molecules is smaller than 7 nm$^{[29]}$. In the present study, the $r$ was smaller than 7 nm at all reaction temperatures, suggesting that non-radioactive energy transfer can quench fluorescence.

**Table 4.** $K$ Values of BLM Helicase at Different Temperatures

<table>
<thead>
<tr>
<th>Quenching agent</th>
<th>T/°C</th>
<th>$\log[(F_0-F)/F] - \log[Q]$</th>
<th>$K$ ($\times 10^4$ L·mol$^{-1}$)</th>
<th>$n$</th>
<th>$R^2$</th>
<th>$R_0$/nm</th>
<th>$r$/nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLRX</td>
<td>4</td>
<td>$y=0.9965 x+6.844$</td>
<td>6.982</td>
<td>0.9965</td>
<td>0.9975</td>
<td>4.01</td>
<td>4.77</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>$y=0.9941 x+6.685$</td>
<td>4.842</td>
<td>0.9941</td>
<td>0.9978</td>
<td>4.09</td>
<td>4.89</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>$y=0.9973 x+6.556$</td>
<td>3.597</td>
<td>0.9973</td>
<td>0.9955</td>
<td>4.16</td>
<td>4.95</td>
</tr>
</tbody>
</table>
Table 5. $\Delta H$, $\Delta G$, and $\Delta S$ of the Reaction between FLRX and BLM$^{642-1290}$ Helicase at Different Temperatures

<table>
<thead>
<tr>
<th>Quenching Agent</th>
<th>T/°C</th>
<th>$K$ ($\times10^5$ L·mol$^{-1}$)</th>
<th>$\Delta G$ (kJ·mol$^{-1}$)</th>
<th>$\Delta H$ (kJ·mol$^{-1}$)</th>
<th>$\Delta S$ (J·mol$^{-1}$·K$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLRX</td>
<td>4</td>
<td>6.982</td>
<td>-36.02</td>
<td>-38.12</td>
<td>-13.15</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>4.842</td>
<td>-39.37</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>3.597</td>
<td>-39.37</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

![Graph 1](image1.png)  
**Figure 11.** $\Delta H$, $\Delta G$, and $\Delta S$ of the reaction between FLRX and BLM$^{642-1290}$ helicase.

![Graph 2](image2.png)  
**Figure 12.** Overlap of BLM helicase fluorescence spectrum (1) and FLRX UV absorption spectrum (2).

**DISCUSSION**

In this study, the effect of FLRX on biological activity and conformation of BLM$^{642-1290}$ helicase and its mechanism were studied by fluorescence polarization, spectroscopy, phosphorus-free assay, and gel retardation assay, which may provide a substantial theoretical basis for full elucidation of the molecular mechanism of DNA helicase as a drug target. Up to date, little information is available on the effect of FLRX on the biological activity of helicase$^{6,8}$. FLRX can increase the DNA unwinding and DNA-binding activity and inhibit the ATPase activity. The reaction between helicase and FLRX can form the BLM-FLRX complex at one binding site between the two molecules. The forces acting on helicase and FLRX include electrostatic force and hydrophobic interaction force. Moreover, FLRX can quench the intrinsic fluorescence of helicase due to non-radioactive energy transfer, as demonstrated in this study.

The biological activity of BLM$^{642-1290}$ helicase was sensitive to FLRX. In this study, FLRX inhibited the DNA unwinding and ATPase activity of helicase *in vitro* in a concentration- and time-dependent manner (Figures 5 and 6). However, the half-inactivated dosage of FLRX inhibiting the DNA unwinding activity ($C_50$ was 60.5±10.2 μmol/L) was about 6-fold higher than that inhibiting the ATPase activity ($C_50$ was 10.3±3.1 μmol/L), suggesting that the ATPase activity is more sensitive to FLRX than the DNA unwinding activity. FLRX promoted DNA-binding activity of helicase. The effect of FLRX on helicase and BLM-DNA complex was significantly different, showing that the action mechanism of FLRX, helicase and BLM-DNA complex is different.

BLM$^{642-1290}$ helicase is consisted of DEAH helicase, RecQ-Ct (RecQ Conserved-Terminal), and HRDC (helicase and RNaseD C-terminal) domains$^{7}$. The DEAH helicase domain is a 7-amino acid sequence motif (directly or indirectly) responsible for binding DNA, inducing ATP hydrolysis, translocation and unwinding$^{32-36}$. The RecQ-Ct domain is located just after the conserved 7 signature motifs and composed of a platform of α-helices with 4 conserved cysteine residues that bind to a Zn$^{2+}$ ion and the winged helix motif$^{33,35-36}$. The DEAH helicase and RecQ-Ct domains are combined to form the catalytic ‘helicase core’ domain, containing the necessary sequence motifs for its ATPase and DNA unwinding activities$^{36}$. The HRDC domain distal to the C-terminus is important in modulating the helicase function via auxiliary contacts to DNA$^{37}$. The distinct functions of the 3 domains may be the reason why ATPase activity is a more sensitive target and FLRX can bind to the catalytic ‘helicase core’ domain. In agreement with this result, the BLM-FLRX complex was formed at binding site due to the electrostatic force and hydrophobic interaction force in this study. However, the reason why FLRX promotes DNA-binding activity is still unclear, and
thus, further study is needed.

The bioactivity of fluoroquinolone drugs mainly depends on the presence of an aromatic fluorine substituent at C-6 due to the enhanced enzyme affinity to substrates. The carboxylic acid at position 3 and the ketone group at position 4 are necessary for inhibition of DNA gyrase activity, whereas substitutions at positions 1 and 7 influence the potency and biological activity of drugs. The carboxylic acid at position 3 and the ketone group at position 4 of fluoroquinolone drugs are the binding sites for enzymes, because the carboxylic acid may provide the hydroxyl and the ketone group contains a coplanar carbonyl group in which hydrogen bonds to enzymes and forms enzyme complexes. The fluoro, chloro, methyl, and methoxy group substituent at C-7 also participate in strengthening the attachment of drug-enzyme-DNA complex. Thus, we may conclude that FLRX can promote the DNA-binding activity of helicase due to the substitutional functional groups at C-6 and C-7 of FLRX, which may enhance the enzyme activity and strengthen the attachment of drug-enzyme-DNA complex. Based on the different functions of the 3 domains, FLRX may bind to the catalytic ‘helicase core’ domain through the carboxylic acid at position 3 and the ketone group at position 4 of FLRX, thus influencing the biological activity. However, the mechanisms as how FLRX affects the 3 activities of helicase require further analysis.

Drugs targeting the biological activity of helicase include one or more of the following mechanisms: inhibition of ATPase activity due to interference with ATP binding and therefore limiting the energy necessary for unwinding and translocation, inhibition of ATPase activity through an allosteric mechanism and stabilizing the enzyme conformation in a low helicase activity state, inhibition of nucleic acids binding to helicase, inhibition of the coupling of ATP hydrolysis to unwinding, inhibition of unwinding by blocking helicase translocation, small molecule antagonists-induced essential protein-protein interactions involving helicases in cells. It is evident that a combination of two or more of these

Figure 13. Inhibitory effect of FLRX on DNA-binding activity of helicase by stabilizing the enzyme conformation (A), disrupting the coupling of ATP hydrolysis to unwinding (B), and blocking the helicase translocation to DNA strands (C).
mechanisms plays an important role in therapy, especially in cancer treatment. In this study, FLRX bonded to the catalytic ‘helicase core’ domain of helicase affected the biological activities of helicase, suggesting that FLRX is a noncompetitive inhibitor for ATPase activity of helicase. Noncompetitive inhibition models a system where the inhibitor and substrate may be bound to the enzyme at any given time. The enzyme-substrate-inhibitor complex cannot be produced but can only be converted back to the enzyme-substrate complex or the enzyme-inhibitor complex when the substrate is bond to the inhibitor. The most common mechanism of noncompetitive inhibition involves reversible binding of the inhibitor to an allosteric site, but it is also possible for the inhibitor to operate via other means, including direct binding to the active site. This is different from competitive inhibition because the binding of the inhibitor does not prevent binding of the substrate and vice versa. Therefore, FLRX promotes the DNA-binding activity of helicase. Furthermore, FLRX can inhibit DNA unwinding and ATPase activity (Figure 13).

Many natural and synthetic compounds with the ability to interact with DNA, developed and used in anticancer therapies, usually perturb DNA structure through electrostatic binding to the phosphoric acid backbone of DNA. The modified DNA structure may inhibit or inactivate DNA-related enzymes in DNA replication, repair, recombination and transcription. For these reasons, we may conclude that FLRX affects DNA by binding to helicase. It was reported that calf thymus DNA can bind to 4 quinolone drugs including FLRX, indicating that FLRX interacts with DNA and affects the DNA structure. Radi et al. reported that the model for binding of quinolone drugs with DNA is established based on the electrostatic binding and intercalation, indicating that FLRX can interact with DNA and bind to helicase by inhibiting the DNA unwinding and ATPase activity, and DNA helicase can be used as a drug target.

REFERENCES


42. Yuan XY, Guo DS, Wang LL. Influence of Mg²⁺ and Cd²⁺ on the interaction between sparfloxacin and calf thymus DNA. Spectrochimica acta part A: molecular and biomolecular spectroscopy, 2008; 64(4), 1130-5.